Published in final edited form as:

Biochemistry. 2000 December 26; 39(51): 16206–16212.

DCCD Inhibits the Reactions of the Iron–Sulfur Protein in *Rhodobacter sphaeroides* Chromatophores[†]

Vladimir P. Shinkarev[‡], Natalia B. Ugulava^{‡,§}, Antony R. Crofts^{||}, and Colin A. Wraight^{*,||}

[‡]Department of Plant Biology, University of Illinois at Urbana–Champaign, 265 Morrill Hall, 505 South Goodwin Avenue, Urbana, Illinois 61801

^{||}Department of Biochemistry, University of Illinois at Urbana–Champaign, 419 Roger Adams Lab, 600 South Mathews Avenue, Urbana, Illinois 61801

Abstract

N,N'-dicyclohexylcarbodiimide (DCCD) has been reported to inhibit proton translocation by cytochrome *bc*₁ and *b₆f* complexes without significantly altering the rate of electron transport, a process referred to as decoupling. To understand the possible role of DCCD in inhibiting the protonogenic reactions of cytochrome *bc*₁ complex, we investigated the effect of DCCD modification on flash-induced electron transport and electrochromic bandshift of carotenoids in *Rb. sphaeroides* chromatophores. DCCD has two distinct effects on phase III of the electrochromic bandshift of carotenoids reflecting the electrogenic reactions of the *bc*₁ complex. At low concentrations, DCCD increases the magnitude of the electrogenic process because of a decrease in the permeability of the membrane, probably through inhibition of F₀F₁. At higher concentrations (>150 μM), DCCD slows the development of phase III of the electrochromic shift from about 3 ms in control preparations to about 23 ms at 1.2 mM DCCD, without significantly changing the amplitude. DCCD treatment of chromatophores also slows down the kinetics of flash-induced reduction of both cytochromes *b* and *c*, from 1.5–2 ms in control preparations to 8–10 ms at 0.8 mM DCCD. Parallel slowing of the reduction of both cytochromes indicates that DCCD treatment modifies the reaction of QH₂ oxidation at the Q_o site. Despite the similarity in the kinetics of both cytochromes, the onset of cytochrome *c* re-reduction is delayed 1–2 ms in comparison to cytochrome *b* reduction, indicating that DCCD inhibits the delivery of electrons from quinol to heme *c*₁. We conclude that DCCD treatment of chromatophores leads to modification of the rate of Q_oH₂ oxidation by the iron–sulfur protein (ISP) as well as the donation of electrons from ISP to *c*₁, and we discuss the results in the context of the movement of ISP between the Q_o site and cytochrome *c*₁.

The cyclic electron transport in chromatophores of non-sulfur purple bacteria includes two main membrane-protein complexes – the photosynthetic reaction center (RC)¹ and cytochrome *bc*₁ complex. Upon illumination, the RC reduces ubiquinone to ubiquinol and oxidizes a water-soluble cytochrome *c*₂. The cytochrome *bc*₁ complex oxidizes ubiquinol and reduces cytochrome *c*₂, with coupled proton release and uptake that contribute to a transmembrane electrochemical gradient of protons (1). Both the reaction center (2–6) and the mitochondrial cytochrome *bc*₁ complex (7–10) have been crystallized and their structures solved to 2.5–3 Å

[†]This work was supported by NIH Grants GM53508 (to C.A.W. and V.P.S.) and GM 35438 (to A.R.C.).

© 2000 American Chemical Society

^{*}To whom correspondence should be addressed. Phone: (217)333-1630. Fax: (217)244-6615. cwraight@uiuc.edu.

[§]Present address: Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104.

¹Abbreviations: EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydro-quinoline; DCCD, *N,N'*-dicyclohexylcarbodiimide; ISP, Rieske iron–sulfur protein; ΔpH, transmembrane pH gradient; RC, photosynthetic reaction center; PMS, *N*-methylphenazonium methosulfate.

atomic resolution. The catalytic core of the bc_1 complex consists of three subunits: cytochrome b , cytochrome c , and an iron–sulfur protein (ISP).

The most surprising feature of the cytochrome bc_1 crystal structures has been the different positions occupied by ISP, depending on the occupation of the Q_o binding site, and different crystal forms (8,9). This suggests that ISP functions via anchored back-and-forth movement of the catalytic domain in order to couple oxidation of ubiquinol and cytochrome c_1 reduction. If the movement occurs through simple 1-D diffusion, calculation indicates that the transitions can occur in the nanosecond time range, and molecular dynamic simulation suggests that there is no structural impediment to such movement (11). When ISP adopts a conformation with the iron–sulfur center close to cytochrome c_1 , the distance between the center and cytochrome c_1 heme is approximately 15 Å (8,10), allowing fast electron transfer between them. Sadoski et al. (12) have used a ruthenium dimer complex to photooxidize cytochrome c_1 , within 1 μ s. In the *Rhodobacter (Rb.) sphaeroides* bc_1 complex, the photooxidized c_1 is reduced by ISP with a lifetime of 17 μ s, similar to the time estimated from previous kinetic work (13).

The proton-motive Q-cycle (refs 14 and 15; reviewed in ref 16), widely accepted as the underlying mechanism of cytochrome bc (and bf) complexes, links electron transfer and vectorial proton transport in an obligatory fashion. However, numerous investigators have reported conditions in which this obligate coupling appears to be violated. Among these are the effects of the covalent carboxyl modifying agents, particularly N,N' -dicyclohexylcarbodiimide (DCCD). It has been shown that the steady-state H^+/e^- ratio can be decreased after modification of the cytochrome bc_1 complex by DCCD (17-23). Treatment of many preparations with DCCD was reported to result in substantial loss of proton release from the “o” side, with only a small effect on the steady-state electron transport by the cytochrome bc complex, a process referred to as decoupling. Several hypotheses have been introduced to explain the decoupling of electron and proton transport by DCCD (reviewed in ref 16;24). According to Brandt and Trumpower (16), DCCD blocks the putative proton channel(s) conducting the proton generated during ubiquinol oxidation at center “o”. This, in turn, forces the proton to move along an artificial pathway through the complex to the other side of the membrane.

Takamiya (25) found that DCCD inhibits electron transfer and electrogenic reactions in the *Rb. sphaeroides* bc_1 complex with similar titers. Wang et al. (26), using isotopically labeled DCCD, suggested that the decoupling effect of DCCD in *Rb. sphaeroides* was due to the modification of aspartate-187 in the cytochrome b subunit. However, we have recently found that the effect of DCCD on the electrochromic bandshift of carotenoids is identical in chromatophores from wild type and mutant cells in which aspartate-187 in cytochrome b has been changed to asparagine (27). Thus, any decoupling effect must be due to chemical modification of another site.

Here we report further studies on the effects of DCCD on the electron transport and electrochromic shift of carotenoids in *Rb. sphaeroides* chromatophores. We found that DCCD effectively inhibits the electron transfer between Q_oH_2 and ISP and between ISP and cytochrome c_1 . The similar titer of DCCD inhibition for electron transport and the electrochromic bandshift indicates the absence of a decoupling effect of DCCD in *Rb. sphaeroides* chromatophores. On the basis of the effect of DCCD on the flash-induced electron transport and electrogenic reactions of the bc_1 complex, we suggest that the decoupling effect in this system observed earlier under steady-state conditions (26) might be simply explained by a slowing of the electrogenic reactions and thus a relative increase of unproductive leakage of the membrane.

MATERIALS AND METHODS

Growth of *Rb. sphaeroides* and Isolation of Chromatophores

Cells of the BC17C strain of *Rb. sphaeroides* Ga with native bc_1 complex expressed in a plasmid were grown aerobically at 30 °C in the dark in Sistrom's medium, in the presence of kanamycin (20 $\mu\text{g}/\text{mL}$) and tetracycline (1.5 $\mu\text{g}/\text{mL}$). Cells were then transferred to semiaerobic conditions at 30 °C in Sistrom's minimal medium enriched with yeast extract, (bacto)tryptone, and casamino acids (Difco, Detroit, MI) and with 1.5 $\mu\text{g}/\text{mL}$ tetracycline as described in ref 28. When grown under these conditions, the cells overexpressed the bc_1 complex so as to yield chromatophores having a relative stoichiometry of about equal concentrations of RCs, cytochrome c_2 , and cytochrome bc_1 complex (cf. 2:1:1 in genomic wild type, ref 15). Before isolation of chromatophores, cells were grown anaerobically at 30 °C 1 day in the light. Cells were disrupted by a single pass through a French press at 18000 psi in the presence of a small amount of DNase. Chromatophores were isolated in 10 mM HEPES (pH 7.5) by differential centrifugation as described elsewhere (29).

Modification of Chromatophores by DCCD

Chromatophores were incubated with aliquots of freshly prepared 400 mM DCCD stock solution in ethanol for 40 min at room temperature. Control samples were treated with the ethanol only. Where indicated, DCCD-treated chromatophores were diluted with 10 vol of 50 mM MOPS/100 mM KCl buffer and spun down for 90 min.

Spectrophotometric Determination of Redox Changes of Cytochromes, Photoactive Pigment, and Electrochromic Shift of Carotenoids

Kinetics of cytochromes and the electrochromic carotenoid bandshift were measured with a single beam kinetic spectrophotometer of local design. Light pulses were delivered by xenon flash (<10 μs half-duration). The flash-induced redox changes of cytochrome c_1 plus cytochrome c_2 were measured at 551–542 nm. The oxidized-minus-reduced extinction coefficient ($\Delta\epsilon$) used for c_{tot} at 551–542 nm was 20 $\text{mM}^{-1} \text{cm}^{-1}$ (30,31). Cytochrome b_H reduction was measured at 561–569 nm in the presence of antimycin, and its concentration was estimated using $\Delta\epsilon = 20 \text{ mM}^{-1} \text{cm}^{-1}$ (15). The concentration of reaction centers was estimated from the flash-induced amplitude at 542 nm using $\Delta\epsilon = 10 \text{ mM}^{-1} \text{cm}^{-1}$ for P870 (30). The electrogenic activity of the cytochrome bc_1 complex was monitored by measuring the slow phase (phase III) of the carotenoid electrochromic bandshift at 503 nm.

Materials

Antimycin A, DCCD, HEPES, and MOPS were obtained from Sigma Chemical Co. Myxothiazol was obtained from Fluka.

RESULTS

Kinetics of Electrochromic Shift and Electron Transport before and after DCCD Treatment

Figure 1 shows the flash-induced electrochromic shift and kinetics of cytochrome c redox reactions for control (panel A) and DCCD-treated (panel B) chromatophores measured at $E_h \approx 100 \text{ mV}$, where the quinone pool is partially reduced before the flash. The kinetics of the carotenoid bandshift in chromatophores have been frequently described in terms of three phases. Phases I and II, completed in less than 200 μs , are attributed to reactions in or close to the RC, and phase III is attributed to reactions of the bc_1 complex (reviewed in ref 32). Treatment of chromatophores with DCCD ($[\text{DCCD}]:[bc_1] = 400$ before washing) leads to the disappearance of phase III of the electrochromic shift, reflecting inhibition of the electrogenic reactions in the bc_1 complex. The cytochrome c rereduction time (τ) is also slowed from about

1.4 (Figure 1A) to 15 ms (Figure 1B) after the treatment. Note that washing of the DCCD-treated chromatophores, which provided for more than 1000 dilution factor, did not reverse the effect of the DCCD modification. These effects of DCCD treatment agree well with similar observations made earlier by Takamiya (25).

Effect of DCCD Modification on the Electrochromic Shift

Figure 2 shows the effect of different DCCD concentrations on the electrochromic bandshift of carotenoids measured at 503 nm. The kinetics of the bandshift are shown on fast (10 ms) and slow (200 ms) time scales. At low concentrations ($<150 \mu\text{M}$), DCCD stimulates the amplitude of phase III and modifies its kinetics, presumably due to the well-characterized blockage of the proton flux through F_o or F_oF_1 by DCCD (33,34). Further increase of the DCCD concentration leads to a significant slowdown of phase III generation and to an apparent acceleration of the decay of the electrochromic shift (compare kinetics at 150 and $700 \mu\text{M}$ DCCD in Figure 2A). The acceleration probably reflects the unmasking of an intrinsic decay due to leakage across the membrane, as the rise kinetics are slowed with increasing inhibition by DCCD. In principle, the progressive inhibition of multiple turnovers could also produce this effect, but at the 1:1:1 stoichiometry for RC: c_2 : bc_1 in these preparations, multiple turnovers are expected to be minimized.

Figure 2B shows phase III alone, which was obtained as the difference between traces with DCCD and traces in the presence of antimycin and myxothiazol. To separate the effect of DCCD on phase III and phases I + II, these differences were taken after normalization of trace amplitudes at 0.2 ms (Figure 2B). The continued rise in phase III, seen on the slower time scale, may include a small degree of multiple turnovers of the bc_1 complex. However, because each curve of phase III in Figure 2B is obtained by subtracting the trace in the presence of antimycin A and myxothiazol, some fraction of this slow rise of phase III also arises from the known weak uncoupling effect of antimycin A (35).

The effect of DCCD on the kinetics of phase III of the electrochromic bandshift are quantified in Figure 3, which shows the dependence of the rate constant (single-exponential fit) and amplitude of phase III of the electrochromic shift on DCCD concentration in chromatophores on a fast time scale. For DCCD concentrations from 100 to $1200 \mu\text{M}$, there is a nearly 10-fold inhibition of the phase III rate without significant change in the amplitude (Figure 3B).

Effect of DCCD Modification on the Redox Reactions of Cytochromes

Figure 4 shows the effect of DCCD on the kinetics of flash-induced cytochrome ($c_2 + c_1$) re-reduction in chromatophores. At $500 \mu\text{M}$ DCCD, the rate of cytochrome c re-reduction was decreased 5-fold with little change in the amplitude.

Figure 5 shows the effect of DCCD on the flash-induced kinetics of the cytochromes in the presence of $10 \mu\text{M}$ antimycin A, an inhibitor of the Q_i binding site. The kinetics are shown on fast and slow time scales to differentiate the kinetics of cytochrome c oxidation (fast time scale) and cytochromes c and b reduction. Within the limitations imposed by the time resolution, the rate and extent of cytochrome c oxidation were unaffected by DCCD treatment, but the rates of cytochrome c re-reduction decreased significantly. The dual time scale also makes clear the existence of a 0.2–0.4-ms delay in cytochrome b reduction (lag phase). Cytochrome b reduction does not begin until the cytochrome c oxidation phase is complete. This has been primarily associated with the kinetics of delivery of an oxidizing equivalent to the Q_o site (15).

Modification of chromatophores with DCCD leads to a substantial and similar slowing of the kinetics of reduction of both cytochromes, without significant changes in the amplitudes. The parallel change in the kinetics of both cytochromes indicates that modification of quinol

oxidation in the Q_o site is the most probable origin of the DCCD effect. Although the rates are similar for the two cytochromes, DCCD treatment introduces a delay in the onset of cytochrome *c* reduction so that it distinctly lags cytochrome *b* reduction (Figure 6). At 800 μ M DCCD, the delay is about 1 ms. We should note that the coincidence of kinetics for cytochromes *b* and *c* reduction is more obvious in these chromatophores because of the overexpression of cytochrome bc_1 complex to RC. At RC: c_2 : bc_1 of 2:1:1, as normally seen in genomic wild type (15), a much smaller fraction of cytochrome *c* is re-reduced after the flash in the presence of antimycin A, and sharing of electrons between ISP, c_2 , and c_1 makes the kinetic match less obvious.

Figure 5B shows also the results of washing the chromatophores after DCCD treatment (with 40 min incubation time). The washing procedure not only fails to reverse the inhibition of cytochromes *b* and *c* reduction, but in fact increases the effect of DCCD, possibly due to the additional incubation time with DCCD before and during the 90-min centrifugation.

DISCUSSION

Effects Induced by DCCD on the Electrochromic Shift of Carotenoids

DCCD has two clearly different effects on the electrochromic shift of carotenoids. At low concentrations, DCCD increases the magnitude of the changes at 503 nm (see Figures 2 and 3). This is likely due to a decrease in the permeability of the membrane through F_o or F_oF_1 after DCCD binding, due to proton leakage. The difference kinetics show that DCCD blocked a leak in a small and variable portion (<25%) of the preparation, with a time constant of about 1.5 ms (Figure 7). This most likely represents a fraction of chromatophores in which a rapid conduction of protons through F_o occurs due to damage to the ATP synthase complex, as is well documented in the literature (33, 34). For the remaining fraction (>75%) and for the entire population once the leak is blocked, further addition of DCCD has no effect on proton conduction.

At higher concentrations (>150 μ M), DCCD inhibits phase III of the electrochromic shift (Figure 2). Single-exponential deconvolution of the kinetics of phase III reveals that for DCCD concentrations from 200 to 1200 μ M, the rate is inhibited about 10-fold but with no significant change in the amplitude. Thus, the visual disappearance of phase III at high DCCD concentrations is due to retardation of the rate of generation. We attribute this inhibition of phase III, induced by DCCD, to a slowing of the rate of QH_2 oxidation by ISP at site “o” (see below).

Effect of DCCD on the Kinetics of Electron Transfer in the bc_1 Complex

(a) Cytochrome *b* Reduction—DCCD slows the kinetics of flash-induced cytochrome *b* reduction without significant changes in the amplitude seen with antimycin A present (Figure 5). The relative constancy of the amplitude of cytochrome *b* reduction indicates that oxidation of QH_2 occurs to completion. The close similarity of the rates of cytochrome *b* reduction and cytochrome *c* re-reduction suggests that the slowdown is due to changes in the binding of QH_2 and/or its oxidation by ISP. Inhibition of the reaction between semiquinone at the “o” site and cytochrome b_L cannot be ruled out, as the reduction of ISP and cytochrome b_L are coupled by the high redox potential of the QH_2/QH couple, but the reactions of the low-potential branch of the bc_1 complex ($Q \rightarrow b_L \rightarrow b_H$) are not detectably modified by the DCCD treatment.

The particular partial reaction of QH_2 oxidation at site “o” that might be inhibited by DCCD is not obvious. Possible targets include QH_2 binding and the docking and undocking of ISP at the “o” site. Also, analysis of mutants of ISP shows a correlation between the midpoint potential (E_m) of ISP and slower cytochrome *b* reduction (39,44), and an effect of DCCD on the E_m is also possible. The manner in which some of these possibilities might slow the kinetics of

cytochrome *b* reduction is dependent on the model envisioned for the “o” site reactions, which is controversial (see, e.g., refs 16 and 39-45).

(b) Cytochrome *c* Reactions—Figure 5 indicates that the rate of cytochrome *c* oxidation (which includes fast equilibration between c_2 , c_1 , and ISP docked near c_1) is not markedly affected by DCCD treatment. Thus, we can exclude the reactions between cytochrome c_1 , c_2 , and ISP^{red} as the main site of inhibition. This is consistent with a conformation for most of the ISP^{red} initially docked near c_1 under the conditions of these experiments (47). Instead, the introduction by DCCD of a significant delay between reduction of cytochrome *b* and cytochrome *c* re-reduction (Figure 6) suggests that DCCD modification affects the reduction of cytochrome c_1 by ISP *after* QH₂ oxidation by ISP.

Both these DCCD effects (slowing of cytochromes *b* and *c* reduction and delay in cytochrome *c* reduction) can be explained by interference of a DCCD adduct with the docking of ISP^{ox} (decrease in rates of cytochromes *b* and *c* reduction) and undocking of ISP^{red} (delay) near cytochrome *b*.

The faster reduction of cytochrome *b* as compared to cytochrome *c* observed here in the DCCD-treated chromatophores is reminiscent of the original difficulties of the Q-cycle in accounting for the imperfect match between cytochromes *b* and cytochrome c_2 (or cytochrome *f*, see ref 36) reductive kinetics. This was explained as arising from the invisible participation of the ISP between the Q_o site and cytochrome *c* (15). The ISP represents an additional sink and can influence both the kinetics and the amplitude of observed electron transfer in the high potential path.

In some mutant b_6f complexes with modified cytochrome *f* (37), the kinetic discrepancy is even more marked, with the reduction of cytochrome b_6 preceding that of cytochrome *f* substantially. It is possible that docking and undocking of ISP to its partners can explain the phenomenon of nonconcerted cytochrome reduction. Thus, mutations in cytochrome *f* may slow the docking of reduced ISP^{red} to cytochrome *f*, causing the reduction of cytochrome *b* to appear relatively faster.

Interestingly, a qualitatively similar effect of EEDQ is seen in the pre-steady-state kinetics of bovine bc_1 complex (23). Reduction of cytochrome *c* was seen to lag cytochrome *b* reduction by 1–2 s.

DCCD Target

Wang et al. (26) showed that ¹⁴C-labeled DCCD substantially modifies cytochrome *b* in *Rb. sphaeroides*. On the basis of peptide digestion, they assigned their decoupling effect of DCCD to specific modification of the surface exposed residue, aspartate-187. However, we recently found that the effect of DCCD on the electrochromic bandshift of carotenoids is identical in chromatophores from wild type and mutant cells in which aspartate-187 in cytochrome *b* has been changed to asparagine, indicating that any DCCD effects must be due to the chemical modification of another site (27). Analysis of the X-ray structural data shows that this residue is not close enough to the Q_o site, or any potential H⁺ channel connected to the site, to have an obvious functional role (24). Thus, we concluded that the observed effects of DCCD are not due to modification of aspartate-187 but might arise from nonspecific modification, possibly at several sites (even non-carboxylic ones, see, e.g., ref 38). This conclusion is also supported by the observation of Cocco et al. (22) that the hydrophobic nucleophile AMF, which requires a carboxyl residue modified by DCCD for formation of an amide bond, did not react with DCCD-treated bovine cytochrome *b*. An attractive alternative is modification of one or more non-carboxylic residues that interfere with ISP movement or even ISP itself. Labeling of ISP in *Rb. sphaeroides* by [¹⁴C]DCCD was not ruled out by Wang et al. (26) and is very

evident in other species (20,21,48). Also, modification of aspartate-166 (bovine numbering) in ISP with EEDQ results in similar effects on the bovine bc_1 complex as DCCD and these have also been described as decoupling (22). This residue is next to Tyr-165, which forms a hydrogen bond to one of the ligand cysteines. Mutations of the residue equivalent to Tyr-165 in *Rb. sphaeroides* can have dramatic effects on E_m , pK , and reaction rate for the Q_0 site reaction (39,44 and references therein).

We previously noted that a single site of action for DCCD would be expected to lead to biphasic kinetics at intermediate levels of modification (27). The smoothly changing nature of all the kinetics described here is more consistent with a progressive accumulation of modifications. The possibility that the effect of DCCD in *Rb. sphaeroides* chromatophores may arise from modification of multiple targets is also strongly indicated by the observed dependence of the time for generation of the phase III (Figure 3) on the DCCD concentration, which is concave upward. Similar dependence is observed for the flash-induced reduction of cytochromes (Figures 4 and 5). If only one group of the bc_1 complex was modified by DCCD, one should expect the apparent lifetime of the generation of phase III (τ^{app}) to depend on DCCD concentration according to

$$\tau^{app} = \frac{\tau_1[DCCD] + \tau_2}{[DCCD] + 1}$$

where τ_1 and τ_2 are the generation time for phase III in DCCD-modified and unmodified bc_1 complexes. However, this equation predicts a convex dependence of the apparent lifetime on DCCD concentration, in qualitative disagreement with the experimentally observed dependence. A concave dependence can arise from accumulation of multiple modifications.

Possible Nature of the Decoupling under Steady-State Conditions

Treatment of many preparations with DCCD is reported to result in substantial loss of proton release from the “o” site, with little effect on the steady-state electron transport by the cytochrome bc_1 complex. This effect was interpreted as blockage of a putative proton channel, forcing the protons to take a route through the complex to the other side of the membrane (16). However, the recently determined structure of chicken bc_1 complex provided little support for this hypothesis (24). Furthermore, the effects of DCCD on the single turnover rates of electron transport and electrogenic reactions (i.e., the carotenoid bandshift) reported here are in apparent contradiction with the steady-state measurements.

The significant inhibition by DCCD of the flash-induced electron-transfer rates found here coincides well with the previous report of Takamiya (25). In contrast, in steady-state measurements little or no change in the steady-state electron transfer was observed (22,23, 26). This disagreement between single turnover and steady-state experiments can be explained by the low sensitivity of the steady-state electron-transfer rate to changes in the intra-protein electron-transfer rates when multiple turnovers are limited by the interaction of the bc_1 complex with exogenous or endogenous substrates. Thus, the time for a single turnover of the bc_1 complex under our conditions (3 ms) is 2 orders of magnitude faster than that (500 ms) found in steady-state measurements for bovine bc_1 complex (23).

Our finding that DCCD has no significant effect on the amplitude of phase III of the electrochromic bandshift of carotenoids (when normalized per fast phases I and II corresponding to the charge separation in RCs) indicates that there is no change in the electrogenicity of the reactions of the bc_1 complex, i.e., no change in the number of charges transported per turnover. Moreover, in all our experiments the kinetics of phase III of the electrochromic shift were parallel to the kinetics of electron transport via the bc_1 complex (see

also ref 25), so that the ratio of electrogenesis to electron transfer remains more or less constant. Thus, we do not see any indication of decoupling during flash-induced turnover of the bc_1 complex.

We note, however, that the apparent “decoupling” effect induced by DCCD under steady-state conditions (26) might be explained by the slowing of the electron-transfer reactions at the Q_o site. Although in some preparations a variable amount of leakiness via F_o channels was blocked by low levels of DCCD, it does not affect passive membrane permeability (17,26). Thus, apparent decoupling might be explained by slowing the transmembrane proton gradient generation against a constant rate of passive leak across the membrane. Increasing the turnover time of the coupled electron transport reaction will increase the chance for unproductive leakage of the membrane and decrease the effective protonogenesis of the bc_1 complex (Figure 2).

This interpretation may explain why chemical modification of different carboxyl residues in different subunits of the bc_1 complex (cytochrome *b*, core protein II, subunit IX, and ISP) and located sometimes on different sides of the membrane (22,46) have similar decoupling effects. To accommodate all these results on chemical modification, one needs only to assume that all these modifications of the bc_1 complex slow down its turnover to the extent that dissipation of the proton transmembrane gradient begins to play a significant role in the coupled steady state.

CONCLUSIONS

To investigate the effect of DCCD on electron transport and proton translocation activities in the cytochrome bc_1 complex, we studied the flash-induced kinetics of electrogenic reactions in *Rb. sphaeroides* chromatophores. Our data show that at different DCCD concentrations the kinetics of phase III follow those of the cytochromes in the bc_1 complex. On the basis of the analysis of the kinetics of cytochromes *c* and *b*, we conclude that DCCD treatment of chromatophores leads to modification of the rate of Q_oH_2 oxidation by ISP as well as the transfer of electrons via ISP to c_1 . The slowing of the electron-transfer reactions at the Q_o site induced by DCCD increases the relative probability of the unproductive leakage of the membrane and may effectively decrease the protonogenesis of the bc_1 complex.

Acknowledgments

We thank Eiji Takahashi for contributions to the early stages of these experiments. We are grateful to Dr. R. B. Gennis for providing the plasmid pBC9, carrying the *fbc* operon of *Rb. sphaeroides*.

References

1. Cramer, WA.; Knaff, DB. Energy Transduction in Biological Membranes: A Textbook of Bioenergetics. Springer-Verlag; New York: 1990.
2. Deisenhofer J, Michel H. EMBO J 1989;8:2149–2170. [PubMed: 2676514]
3. Allen JP, Feher G, Yeates TO, Komiya H, Rees DC. Proc Natl Acad Sci U S A 1987;84:6162–6166. [PubMed: 2819866]
4. El-Kabbani O, Chang CH, Tiede D, Norris J, Schiffer M. Biochemistry 1991;30:5361–5369. [PubMed: 2036405]
5. Ermler U, Fritzsche G, Buchanan SK, Michel H. Structure 1994;2:925–936. [PubMed: 7866744]
6. Deisenhofer J, Epp O, Sinning I, Michel H. J Mol Biol 1995;246:429–457. [PubMed: 7877166]
7. Xia D, Yu CA, Kim H, Xia JZ, Kachurin AM, Zhang L, Yu L, Deisenhofer J. Science 1997;277:60–66. [PubMed: 9204897]
8. Zhang Z, Huang L, Shulmeister VM, Chi YI, Kim KK, Hung LW, Crofts AR, Berry EA, Kim SH. Nature 1998;392:677–684. [PubMed: 9565029]

9. Kim H, Xia D, Yu CA, Xia JZ, Kachurin AM, Zhang L, Yu L, Deisenhofer J. *Proc Natl Acad Sci U S A* 1998;95:8026–8033. [PubMed: 9653134]
10. Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, Ramaswamy S, Jap BK. *Science* 1998;281:64–71. [PubMed: 9651245]
11. Izrailev S, Crofts AR, Berry EA, Schulten K. *Biophys J* 1999;77:1753–1768. [PubMed: 10512801]
12. Sadoski RC, Engstrom G, Tian H, Zhang L, Yu C, Yu L, Durham B, Millett F. *Biochemistry* 2000;39:4231–4236. [PubMed: 10757970]
13. Crofts AR, Wang Z. *Photosynth Res* 1989;22:69–87.
14. Mitchell P. *J Theor Biol* 1976;62:327–367. [PubMed: 186667]
15. Crofts AR, Meinhardt SW, Jones KR, Snozzi M. *Biochim Biophys Acta* 1983;723:202–218.
16. Brandt U, Trumpower B. *Crit Rev Biochem Mol Biol* 1994;29:165–197. [PubMed: 8070276]
17. Beattie DS, Villalobo A. *J Biol Chem* 1982;257:14745–14752. [PubMed: 6294076]
18. Lenaz G, Esposti MD, Castelli GP. *Biochem Biophys Res Commun* 1982;105:589–595. [PubMed: 6284158]
19. Price BD, Brand MD. *Biochem J* 1982;206:419–422. [PubMed: 6293466]
20. Lorusso M, Gatti D, Boffoli D, Bellomo E, Papa S. *Eur J Biochem* 1983;137:413–420. [PubMed: 6319124]
21. Nalecz MJ, Casey RP, Azzi A. *Biochim Biophys Acta* 1983;724:75–82. [PubMed: 6307355]
22. Cocco T, Paola MD, Papa S, Lorusso M. *FEBS Lett* 1999;456:37–40. [PubMed: 10452525]
23. Cocco T, Paola MD, Papa S, Lorusso M. *Biochemistry* 1998;37:2037–2043. [PubMed: 9485330]
24. Crofts AR, Guergova-Kuras M, Huang L, Kuras R, Zhang Z, Berry EA. *Biochemistry* 1999;38:15791–15806. [PubMed: 10625445]
25. Takamiya KI. *J Biochem* 1983;94:1587–1594. [PubMed: 6317667]
26. Wang YD, Obungu V, Beattie DS. *Arch Biochem Biophys* 1998;352:193–198. [PubMed: 9587406]
27. Shinkarev VP, Ugulava NB, Takahashi E, Crofts AR, Wraight CA. *Biochemistry* 2000;39:14232–14237. [PubMed: 11087372]
28. Takahashi E, Wraight CA. *Biochemistry* 1992;31:855–866. [PubMed: 1731944]
29. Drachev LA, Kaurov BS, Mamedov MD, Mulkidjanian AJa, Semenov AYU, Shinkarev VP, Skulachev VP, Verkhovsky MI. *Biochim Biophys Acta* 1989;973:189–197.
30. Bowyer JR, Meinhardt SW, Tierney GV, Crofts AR. *Biochim Biophys Acta* 1981;635:167–186. [PubMed: 6260162]
31. Meinhardt SW, Crofts AR. *FEBS Lett* 1982;149:223–227.
32. Jackson, JB. *Bacterial Energy Transduction*. Anthony, C., editor. Academic Press; New York: 1988. p. 317–375.
33. Saphon S, Jackson JB, Witt HT. *Biochim Biophys Acta* 1975;408:67–82. [PubMed: 240444]
34. Feniouk BA, Cherepanov DA, Junge W, Mulkidjanian AY. *FEBS Lett* 1999;445:409–414. [PubMed: 10094498]
35. Oleskin AV, Samuilov VD. *Biochemistry (Moscow)* 1988;53:1803–1809.
36. Selak MA, Whitmarsh J. *FEBS Lett* 1982;150:286–292.
37. Ponamarev MV, Cramer WA. *Biochemistry* 1998;37:17199–17208. [PubMed: 9860833]
38. Nalecz MJ, Casey RP, Azzi A. *Methods Enzymol* 1986;125:86–108. [PubMed: 2872577]
39. Guergova-Kuras M, Kuras R, Ugulava N, Hadad I, Crofts AR. *Biochemistry* 2000;39:7436–7444. [PubMed: 10858292]
40. Brandt U. *Biochim Biophys Acta* 1998;1365:261–268. [PubMed: 9693740]
41. Link TA. *FEBS Lett* 1997;412:257–264. [PubMed: 9256231]
42. Sharp RE, Gibney BP, Palmitessa A, White JL, Dixon JA, Moser CC, Daldal F, Dutton PL. *Biochemistry* 1999;38:3440–3446. [PubMed: 10079091]
43. Hong S, Ugulova N, Guergova-Kuras M, Crofts AR. *J Biol Chem* 1999;274:33931–33944. [PubMed: 10567355]
44. Snyder CH, Gutierrez-Cirlos EB, Trumpower BL. *J Biol Chem* 2000;275:13535–13541. [PubMed: 10788468]

45. Heiman S, Ponamarev MV, Cramer WA. *Biochemistry* 2000;39:2692–2699. [PubMed: 10704220]
46. Beattie DS. *J Bioenerg Biomembr* 1993;25:233–244. [PubMed: 8394319]
47. Crofts AR, Hong S, Zhang Z, Berry EA. *Biochemistry* 1999;38:15827–15839. [PubMed: 10625447]
48. Wang Y, Beattie DS. *Arch Biochem Biophys* 1991;291:363–370. [PubMed: 1659325]

Figure 1.

Flash-induced kinetics of the electrochromic shift of carotenoids (upper curve) and redox reactions of cytochromes *c* (lower curve) in *Rb. sphaeroides* chromatophores at low redox potential ($E_h = 100$ mV) under anaerobic conditions without (A) and with DCCD treatment ([DCCD]:[bc] = 400, panel B). Control samples (A) were treated with the ethanol only. After 40-min incubation with ethanol (A) or DCCD (B), chromatophores were washed with 10 vol of 50 mM MOPS/100 mM KCl buffer and then spun down for 90 min. Conditions: 50 mM MOPS, pH 7.5, 100 mM KCl; 2 μ M 1,2-naphthoquinone and 2 μ M PMS used as redox mediators; 1 mM Fe(III)EDTA present as redox buffer. Traces shown were the average of three separate traces, with 60 s between measurements. Curves were smoothed by 2-point averaging.

Figure 2.

Effect of DCCD on the kinetics of phase III of the electrochromic carotenoid bandshift in chromatophores. (A) Kinetics of the electrochromic carotenoid bandshift in chromatophores, measured at different DCCD concentrations (μM , indicated by numbers near curves). Bottom trace is in the presence of antimycin A ($10\ \mu\text{M}$) and myxothiazol ($5\ \mu\text{M}$). After DCCD additions, the chromatophores were adapted for 30–40 min at room temperature before measurement. $[\text{DCCD}]:[\text{bc}_1] = 50$ for $100\ \mu\text{M}$ DCCD. (B) Phase III of the electrochromic shift of carotenoids in chromatophores, obtained by subtraction of the trace in the presence of antimycin and myxothiazol from the traces at different DCCD concentrations. Before subtraction, the traces were normalized at 0.2 ms to minimize the differences in the amplitude of phases I and II of the electrochromic carotenoid bandshift. Conditions: 50 mM MOPS, pH 7.5, 100 mM KCl; 2 μM 1,2-naphthoquinone and 2 μM PMS used as redox mediators; 1 mM Fe(III)EDTA present as redox buffer. Shown are single traces (without averaging). The instrument response time

was 10 μ s. Curves were smoothed by 2-point (fast time scale) and 5-point (slow time scale) averaging.

Figure 3.

Dependence of the rise time (A) and amplitude (B) of phase III of the electrochromic bandshift on DCCD concentration in *Rb. sphaeroides* chromatophores, obtained from single-exponential fits of the kinetics similar to those shown on a fast time scale in Figure 2B.

Figure 4.

Effect of DCCD concentration (in μM , indicated by numbers near curves) on the kinetics of cytochrome *c* rereduction in *Rb. sphaeroides* chromatophores. Dependence of the lifetime (B) and amplitude (C) of the kinetics of cytochrome ($c_2 + c_1$) re-reduction on DCCD concentration, obtained from single-exponential fits of the kinetics shown in panel A. Conditions: 50 mM MOPS, pH 7.5, 100 mM KCl; 2 μM 1,2-naphthoquinone and 2 μM PMS used as redox mediators; 1 mM Fe(III)EDTA present as redox buffer. After DCCD additions, the chromatophores were adapted for 30–40 min at room temperature before measurement. $[\text{DCCD}]:[bc_1] = 186$ for 500 μM DCCD. Traces shown were the average of three separate traces, with 60 s between measurements. The instrument response time was 50 μs . Curves were smoothed by 5-point averaging.

Figure 5.

Effect of DCCD modification on the kinetics of cytochromes *b* and *c* in the presence of antimycin A (A). Dependence of the lifetime (B) and amplitude (C) of cytochrome *c* ($=c_2 + c_1$) and cytochrome *b* reduction on DCCD concentration, obtained from single-exponential fits of the kinetics shown in panel A. $[\text{DCCD}]:[bc_1] = 100$ for 400 μM DCCD. Diamond and inverted triangle show the lifetime of cytochromes *b* and *c* reduction in chromatophores washed after DCCD treatment. Conditions: 50 mM MOPS, pH 7.5, 100 mM KCl; 2 μM 1,2-naphthoquinone and 2 μM PMS used as redox mediators; 1 mM Fe(III)EDTA present as redox buffer. Traces shown in panel A were the average of three separate traces, with 60 s between measurements. The instrument response time was 10 μs . Curves were smoothed by 2-point averaging.

Figure 6.

Kinetics of the flash-induced cytochromes *b* and *c* reduction in the absence (A) and presence (B) of 800 μ M DCCD. The amplitudes of cytochrome *b* and cytochrome *c* reduction phases were normalized. Conditions: 50 mM MOPS, pH 7.5, 100 mM KCl; 10 μ M antimycin A; 2 μ M 1,2-naphthoquinone and 2 μ M PMS used as redox mediators; 1 mM Fe(III)EDTA present as redox buffer. [DCCD]:[*bc*₁] = 100 for 400 μ M DCCD.

Figure 7.

Difference of the electrochromic carotenoid bandshift at $150 \mu\text{M}$ DCCD and bandshift at zero DCCD concentration, obtained from the curves shown in Figure 2. Before subtraction, the traces were normalized at 0.2 ms to minimize the differences in the amplitude of phases I + II of the electrochromic carotenoid bandshift.